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Leukocyte Chemoattractant Peptides from the Serpin Heparin Cofactor II*

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Frank C. Church‡, Charlotte W. Pratt, and Maureane Hoffman

From the Departments of Pathology and Medicine and The Center for Thrombosis and Hemostasis, The University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

Heparin cofactor II (HC) is a plasma serine proteinase inhibitor (serpin) that inhibits the coagulant proteinase α-thrombin. We have recently demonstrated that proteolysis of HC by catalytic amounts of polymorphonuclear leukocyte proteinases (elastase or cathepsin G) generates leukocyte chemotaxins (Hoffman, M., Pratt, C. W., Brown, R. L., and Church, F. C. (1989) *Blood* 73, 1682–1685). One of four peptides produced when HC is degraded by neutrophil elastase has chemotactic activity for both monocytes and neutrophils with maximal migration comparable to formyl-Met-Leu-Phe, the "gold standard" bacterially derived chemotaxin. The amino-terminal sequence of this HC peptide is Asp-Phe-His-Lys-Glu-Asn-Thr-Val-... and the peptide corresponds to Asp-39 to Ile-66 of HC. A variety of synthetic peptides derived from this sequence were evaluated for leukocyte migration activity, and a dodecapeptide from Asp-49 to Tyr-60 (Asp-Trp-Ile-Pro-Glu-Gly-Glu-Glu-Asp-Asp-Asp-Tyr) was identified as the active site for leukocyte chemotactic action. The 12-mer synthetic peptide possesses significant neutrophil chemotactic action at 1 nM (60% of the maximal activity of formyl-Met-Leu-Phe), while a peptide with the reverse sequence has essentially no chemotactic activity. Cross-desensitization experiments also show that pretreatment of neutrophils with a 19-mer peptide (Asn-48 to Ile-66) greatly reduces subsequent chemotaxis to HC-neutrophil elastase proteolysis reaction products. When injected intraperitoneally in mice, the HC-neutrophil elastase digest elicits neutrophil migration.

Our results demonstrate that not only does HC function as a thrombin inhibitor, but that limited proteolysis of HC near the amino terminus yields biologically active peptide(s) which might participate in inflammation and in wound healing and tissue repair processes.

Serpins are a protein superfamily of which many members possess potent activity as serine proteinase inhibitors (1, 2). Interestingly, a number of serpins are not proteinase inhibitors, including angiotensinogen and thyroxine- and cortisol-binding globulins found in human plasma, ovalbumin from chicken egg white, and endosperm protein Z from barley (1–3). Two plasma serpins, heparin cofactor II (HC)[1] and antithrombin (AT), inhibit the blood coagulation proteinase α-thrombin in a reaction that is dramatically accelerated by heparin and other glycosaminoglycans/proteoglycans (4–8). Heparan sulfate proteoglycans localized to vessel walls may be the primary site of *intravascular* proteinase inhibition mediated by AT, while basement membrane/extracellular matrix dermatan sulfate proteoglycans might be important for HC-mediated thrombin inhibition *extravascularly* (6–10).

Human polymorphonuclear leukocytes (neutrophils) and mononuclear phagocytes (monocytes) are important cellular components of tissue injury, infection, establishment of acute/chronic inflammation, and wound healing (11, 12). Proteolytic enzymes and oxidants are released during activation of neutrophils (11, 12). Limited cleavage of HC by either purified neutrophil elastase or cathepsin G or live activated neutrophils releases peptides with discrete biological activity for leukocytes (13–16). Neutrophil proteinases might generate biologically active peptides from HC without affecting antithrombin inhibitory action (16). A potential physiological role for HC unrelated to proteinase inhibition may depend on the generation of peptides that modulate leukocyte functions.

We are interested in understanding the functional and structural roles of HC compared to AT and other serpins. The carboxyl termini of most serpins are homologous and are the portions of the molecules involved in proteinase inhibition (1). However, the amino terminus of HC has little homology to other serpins, including AT (7, 17). Therefore, we hypothesized that HC may be related in function to the serpin homologue angiotensinogen, whose amino terminus generates bioactive peptides following limited proteolysis by renin and neutrophil proteinases (18, 19).

We report here that limited neutrophil elastase proteolysis of HC produces a peptide derived from the amino-terminal region (corresponding to Asp-39 to Ile-66) with chemoattractant action for both neutrophils and monocytes. We also found that a dodecapeptide from Asp-49 to Tyr-60 of HC may contain the minimal active site for leukocyte migration activity. Additionally, we found that the products of an HC-neutrophil elastase digest stimulate neutrophil migration *in vivo* in mice. Collectively, our data further implicate a role for HC-neutrophil proteinase products in local inflammatory responses. A preliminary report has appeared in abstract form (20).

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‡ To whom correspondence and reprint requests should be addressed: Div. of Hematology, Campus Box 7035, 416 Burnett-Womack, University of North Carolina, Chapel Hill, NC 27599. Fax: 919-966-7639.

[1] The abbreviations used are: HC, heparin cofactor II; AT, antithrombin; fMLP, *N*-formyl-Met-Leu-Phe; hpf, high power fields; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography; DMEM, Dulbecco's modified Eagle medium; α_1-PI, α_1-proteinase inhibitor.

EXPERIMENTAL PROCEDURES

Materials—HC and AT were purified from human plasma as described (17). Human polymorphonuclear leukocyte elastase purified from sputum was obtained from Elastin Products (Pacific, MO) and stored in 200 mM sodium acetate at pH 5.0 at −20 °C. Phenylmethylsulfonyl fluoride and fMLP were from Sigma. All HC/AT proteolysis and HC synthetic peptide experiments were performed in a buffer that contained 20 mM HEPES, 150 mM NaCl, 0.1% (w/v) polyethylene glycol (M_r = 8000) at pH 7.4.

Serpin-Neutrophil Elastase Reactions and HPLC—A mixture of HC and neutrophil elastase (2 μM HC, 20 nM proteinase) was kept at room temperature for 40 min at pH 7.4 in the HEPES buffer. Phenylmethylsulfonyl fluoride (1 mM) was added to terminate proteolysis. The digested sample was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and, as visualized, all of the native HC (M_r ~ 66,000) was converted to a degradation product previously termed "band III" (M_r ~ 48,000; Ref. 16). The HC-neutrophil elastase digest (~100 μg of HC) was chromatographed on a Phenomenex W-Porex C-18 column (4.6-mm inner diameter × 250 mm, with 5-μm particles) with the column equilibrated in 0.1% trifluoroacetic acid, and a gradient was developed with 2-propanol to 22% in 57 min and then to 60% in 34 min at a flow rate of 1 ml/min as described previously (21). The amino acid sequence of peak 2 (see Figs. 1 and 2) was determined on an Applied Biosystems 470A gas-phase sequenator with an on-line Waters HPLC.

Reaction of AT and prothrombin with neutrophil elastase and cathepsin G was performed using the conditions for HC-neutrophil proteinase mixtures as detailed previously (13).

Cell Isolation and Chemotaxis Assay—Monocytes and neutrophils were purified on Ficoll-Hypaque density gradients (Mono-Poly resolving medium, Flow Laboratories) from the blood of healthy volunteers as described previously (13, 14). The purity of the resulting cell preparations was determined by performing differential counts of Wright's-stained cytospin preparations. The neutrophil preparations were routinely 95–99% pure while the mononuclear preparations routinely contained fewer than 5% neutrophils and eosinophils and about 40–50% monocytes, with the remaining cells being lymphocytes.

Chemotaxis was measured in modified Boyden chambers as detailed previously (13). Leukocytes were suspended at 1.5 × 10^6 cells/ml in Dulbecco's modified Eagle medium (DMEM, GIBCO) supplemented with 1 mg/ml dextrose, and containing 10% calf serum (Sterile Systems, Inc.). All buffers, media, and sera used during the isolation of cells and performance of assays contained <1 ng/ml endotoxin. To the top well of each Boyden chamber was added 0.25 ml of the leukocyte suspension. The chemoattractant solution to be tested, or medium alone, was simultaneously added to the bottom compartment. The compartments of the Boyden chambers were separated by a polycarbonate filter with 5-μm pores (Nuclepore, Inc.). The chambers were incubated for 1 h (neutrophils) or 2 h (monocytes) at 37 °C in a humidified atmosphere containing 5% CO_2. The filters were fixed in 5% formalin, stained with Wright's stain, mounted in immersion oil under coverslips, and the number of leukocytes which had migrated across the filter was counted in 10–20 high power fields (hpf) per filter at × 1000 final magnification. The average number of leukocytes migrating in control chambers (lower compartment contained medium only) was subtracted as background from the number migrating in test chambers, to yield the net number of leukocytes migrating per oil field. Positive controls assessing chemotaxis to fMLP (10^{-6} to 10^{-9} M) were conducted in parallel with the experimental groups. Chemotaxis to a test substance was expressed as a percentage of the maximal chemotaxis to fMLP in the same experiment. Migration to each concentration of each potential chemotaxin was tested a minimum of three times.

Peptide Synthesis—Peptides were assembled using a Milligen Pepsynthesizer (with Fmoc (*N*-(9-fluorenyl)methoxycarbonyl)-amino acid chemistry following the guidelines provided by the manufacturer) according to the reported cDNA sequence for HC (7). Purity of the peptides was analyzed by reverse-phase HPLC, and amino acid analysis results were consistent with the theoretical yield.

The α- and ε-amino groups of the 22-mer synthetic HC peptide were modified with acetic anhydride (22). The modified peptide was separated from other reagents using Sephadex G-15 equilibrated in 100 mM ammonium bicarbonate. The extent of amino group modification was determined spectrophotometrically (23).

Electrophoresis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out at 25 °C using 10% polyacrylamide gels as described (24).

Migration Activity for Mouse Neutrophils in Vivo—C3H/HeJ mice (8 weeks old) were injected intraperitoneally through a 0.2-μm filter, with 1 ml of either DMEM alone (with 10% neonatal bovine serum), or with 1 ml of a 0.1 μM solution of HC-neutrophil elastase digest (13) or HC alone in DMEM with neonatal bovine serum. One hour after injection, peritoneal cells were recovered by lavage with DMEM with 10% neonatal bovine serum and 10 units/ml heparin. The cells were sedimented, counted in the Coulter counter, and cytopsins made of the harvested cells and stained with Wright's stain. Differential counts of the harvested cells were done under an oil immersion microscope. *In vivo* migration experiments were each performed three times.

RESULTS

Isolation of a Unique Chemotactic Peptide following Limited Neutrophil Elastase Proteolysis of HC—The peptides produced when HC is degraded by catalytic amounts of neutrophil elastase were purified by reverse-phase HPLC and tested for chemotactic activity (Fig. 1). Of the four peaks separated by HPLC, only peak 2 was found to have significant chemotactic activity for both neutrophils and monocytes. Primary structural analysis of this peptide revealed a sequence of Asp-Phe-His-Lys-Glu-Asn-Thr-Val-. . . . Amino acid analysis of this peptide matches the sequence in HC from Asp-39 to Ile-66 (data not included). It is not known whether the chemotactic activity generated from HC by cathepsin G is localized to this same region; however, cathepsin G also hydrolyzes HC at the Ile^{66}-Phe bond (16). These results indicate that neutrophil elastase cleaves HC into several fragments, but there is only one distinct peptide generated from the amino terminus that possesses leukocyte chemotactic action.

Synthetic HC Peptides Mimic the Chemotactic Action of the HC-Neutrophil Elastase Digest—To determine whether part or all of the sequence contained within Asp-39 to Ile-66 of HC is required for chemotactic activity, we prepared synthetic peptides spanning this region (sequences are shown in Fig. 2) and measured their chemotactic potential (shown in Fig. 3 for neutrophils): from Asp-39 to Asn-48 (10-mer peptide), Asn-48 to Ile-66 (19-mer peptide), Asp-39 to Tyr-60 (22-mer peptide), and the entire Asp-39 to Ile-66 sequence (28-mer peptide). As found for the HPLC-purified HC fragment composed of residues 39–66, the synthetic 28-mer peptide was an effective chemotaxin for neutrophils (60% of fMLP at 1 × 10^{-11} M 28-mer peptide; data not shown). The 22-mer peptide had chemotactic activity (at nanomolar concentrations) for both monocytes (40% of fMLP) and neutrophils (38% of fMLP), as did the 19-mer peptide for monocytes (30% of fMLP) and neutrophils (27% of fMLP), but the 10-mer peptide was inactive (6% of fMLP) (Fig. 3). Interestingly, acetylation of the α- and ε-amino groups of the 22-mer peptide did not diminish leukocyte chemotactic activity compared to the sham-modified 22-mer peptide (data not shown). Leukocyte chemotaxis to a synthetic peptide from another region of HC from Phe-183 to Arg-200 (18-mer peptide) gave negative results (Fig. 3). These results show that the 19-mer and 22-mer peptides mimic the bioactivity of the Asp-39 to Ile-66 HC peptide, and we conclude that the dodecapeptide sequence from Asp-49 to Tyr-60 (Asp-Trp-Ile-Pro-Glu-Gly-Glu-Glu-Asp-Asp-Asp-Tyr) may constitute the minimal active region.

To further assess the ability of the region contained within Asp-49 to Tyr-60 of HC to elicit leukocyte chemotaxis, we assembled the 12-mer peptide based on its correct sequence and a synthetic peptide based on a reverse sequence (r-12-mer; sequence shown in Fig. 2). Elution of the 12-mer and r-12-mer peptides was identical when analyzed by FLPC ion-exchange chromatography (Fig. 4). However, the 12-mer pep-

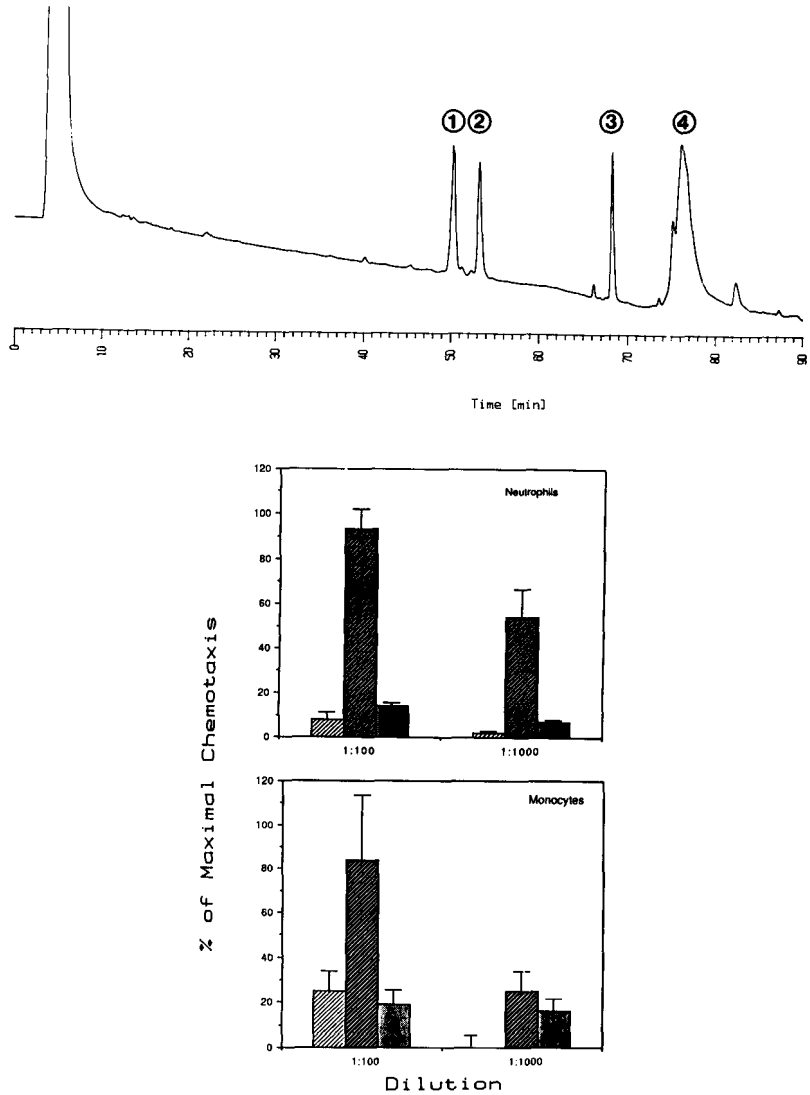

FIG. 1. **Neutrophil elastase proteolysis of HC, peptide purification by HPLC, and leukocyte chemotactic activity of peptides.** *Top,* the HC-neutrophil elastase limited proteolysis digest was prepared and analyzed by reverse-phase HPLC as described under "Experimental Procedures." Shown here is the 210-nm absorbance profile of the digest, where absorbance full scale is 0.25. Each peak was collected and then lyophilized to remove any residual solvent. *Bottom,* redissolved peptides (in a total volume of 1 ml in the HEPES buffer) were measured for their leukocyte chemotactic activity as described under "Experimental Procedures": peak 1 (▨), peak 2 (▨), and peak 3 (▩). The results are expressed at a 1:100 and 1:1000 dilution of the redissolved peaks (peaks 1–3) as the net number of either neutrophils or monocytes migrating per × 1000 field as a percentage of the maximal migration of fMLP (29.5 neutrophils/hpf; 13.4 monocytes/hpf). Each *point* represents the mean of duplicate determinations, and the *bars* represent the range of counts. Peak 4 was not chemotactic for neutrophils and was not further evaluated (data not shown). Control solutions (following lyophilization and redissolution) at the same trifluoroacetic acid/2-propanol mixture at which each peak eluted from the HPLC column were also tested and showed no chemotactic activity (<10% of fMLP).

Peptide	Inhibitor Region	Sequence
28-mer	Asp 39-Ile 66	H-Asp-Phe-His-Lys-Glu-Asn-Thr-Val-Thr-Asn-Asp-Trp-Ile-Pro-Glu-Gly-Glu-Glu-Asp-Asp-Asp-Tyr-Leu-Asp-Leu-Glu-Lys-Ile-OH
10-mer	Asp 39-Asn 48	H-Asp-Phe-His-Lys-Glu-Asn-Thr-Val-Thr-Asn-(Tyr)-OH
19-mer	Asn 48-Ile 66	H-Asn-Asp-Trp-Ile-Pro-Glu-Gly-Glu-Glu-Asp-Asp-Asp-Tyr-Leu-Asp-Leu-Glu-Lys-Ile-(Tyr)-OH
22-mer	Asp 39-Tyr 60	H-Asp-Phe-His-Lys-Glu-Asn-Thr-Val-Thr-Asn-Asp-Trp-Ile-Pro-Glu-Gly-Glu-Glu-Asp-Asp-Asp-Tyr-OH
12-mer	Asp 49-Tyr 60	H-Asp-Trp-Ile-Pro-Glu-Gly-Glu-Glu-Asp-Asp-Asp-Tyr-OH
r-12-mer	Asp 49-Tyr 60 (reverse direction)	H-Asp-Asp-Asp-Glu-Glu-Gly-Glu-Pro-Ile-Trp-Asp-(Tyr)-OH

FIG. 2. **Amino acid sequences of HC peptides.**

tide possessed significant neutrophil chemotactic action (60% of fMLP at 1 nM 12-mer peptide) while the r-12-mer peptide was essentially inactive as a chemotaxin (<10% of fMLP at 10^{-11} to 10^{-7} M r-12-mer peptide) (Fig. 4). These results suggest that the leukocyte chemotactic action of HC peptides (containing Asp-49 to Tyr-60) is dependent upon a unique structural determinant derived from its amino acid sequence and this activity is not dependent upon interactions based solely on charge.

The ability of the 19-mer HC peptide to desensitize neutrophils to further chemotactic action was examined. Exposure of neutrophils to fMLP down-regulates the fMLP receptor and desensitizes the cells to subsequent exposure to fMLP (25). Neutrophils preincubated with the 19-mer peptide did not migrate to a gradient of the HC-neutrophil elastase digest on subsequent testing (Table I). We also found that the 19-mer peptide and fMLP did not cross-desensitize, indicating that the HC-derived chemotaxin does not bind to the fMLP receptor (Table I). These results further indicate that the specific chemotactic activity generated from an HC-neutrophil elastase digest is primarily contained within the Asp-39 to Ile-66 peptide.

Limited Proteolysis of Antithrombin and Prothrombin with Neutrophil Proteinases—Unlike HC, no significant neutrophil chemotactic activity was generated by the reaction of AT with either neutrophil elastase or cathepsin G (Table II). Electrophoretic analysis of the AT/neutrophil elastase reaction products verified previous observations of a cleavage site about 5 kDa from the carboxyl terminus in AT with no apparent amino-terminal degradation (26, 27). While AT is rapidly inactivated by exposure to neutrophil proteinases, HC is initially degraded at the amino terminus to a species that retains thrombin inhibitory activity, further suggesting different functional roles for the amino termini of the two serpins (16, 26–28). Additionally, limited digestion of prothrombin by the neutrophil proteinases resulted in multiple peptides dur-

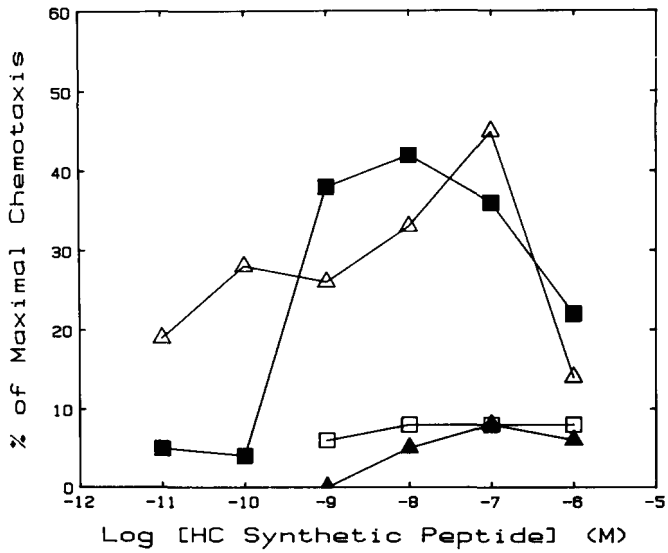

FIG. 3. **Neutrophil chemotactic activity of synthetic peptides derived from the region of Asp-39 to Ile-66 in HC.** Chemotaxis to dilutions of the reaction mixtures was assayed as described under "Experimental Procedures" for the 10-mer peptide (□), 19-mer peptide (△), and 22-mer peptide (■) from Asp-39 to Ile-66, and the 18-mer peptide (▲) from the region of Phe-183 to Arg-200. The concentration of chemoattractant in the lower wells of the Boyden chambers is expressed on the *x*-axis as the logarithm of the molar concentration of each individual synthetic peptide. The results are expressed as the net number of neutrophils migrating per × 1000 field as a percentage of the maximal migration to fMLP (34.9 ± 14.5 neutrophils/hpf). Each *point* represents the mean of at least two separate experiments performed in duplicate.

ing the 5-min incubation (data not included) but without generation of any dramatic leukocyte chemotactic activity (Table II). The absence of formation of leukocyte bioactive peptides from AT and prothrombin (another common plasma protein) following neutrophil proteinase digestion implies that the generation of leukocyte bioactive peptides from HC may be a unique event.

In Vivo Chemotactic Activity of the HC-Neutrophil Elastase Digest—The ability of an HC-neutrophil elastase digest to stimulate neutrophil migration *in vivo* was addressed by injecting mice intraperitoneally, and quantifying neutrophils in the exudate. The HC-neutrophil elastase digest (at 0.1 μM) demonstrated neutrophil migration action *in vivo* compared to the same amount of HC alone or to medium alone (Table III). Control experiments verified that isolated mouse neutrophils migrated in a similar manner to human neutrophils with HC-neutrophil elastase digests and fMLP (data not included). These observations imply that the proteolysis products of the HC-neutrophil elastase digests induce neutrophil migration *in vivo*.

DISCUSSION

This study was undertaken to identify the leukocyte chemoattractant site in HC. Treatment of purified human HC with isolated human neutrophil elastase, with cathepsin G, or with live stimulated neutrophils produces HC fragments with potent effects on leukocyte function (13–16). Our results demonstrate that HC is proteolyzed by neutrophil elastase under physiological conditions to release a peptide composed of Asp-39 to Ile-66. It is not known whether the sulfated tyrosyl residue (29) potentially present in HC and the biologically derived peptide (at residue 60) augments chemotaxis. Nonetheless, synthetic peptides based on this region in HC with nonsulfated tyrosyl residues were quite effective leukocyte

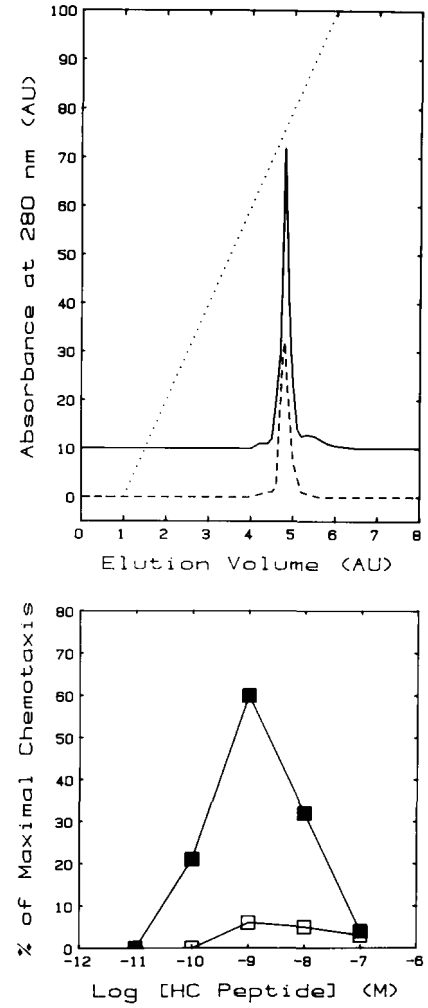

FIG. 4. **FPLC and neutrophil chemotaxis of the 12-mer and r-12-mer HC synthetic peptides.** *Top,* the peptides were applied to a Mono Q (HR5/5) column at a flow rate of 1 ml/min in 20 mM ammonium acetate, pH 8.0, at room temperature. After washing for 2 min, the 12-mer (*solid line*) and the r-12-mer (*dashed line*) were eluted using a linear gradient from 20 mM to 4 M ammonium acetate, pH 8.0. Shown here is the 280-nm absorbance profile in arbitrary units (*AU*). *Bottom,* neutrophil chemotactic activity of the 12-mer (■) and r-12-mer (□). The results are expressed as the net number of neutrophils migrating per × 1000 field as a percentage of the maximal migration of fMLP (38.8 ± 13.4 neutrophils/hpf). Each *point* represents the mean of at least three separate experiments performed in duplicate.

chemotaxins. A dodecapeptide sequence encoded by Asp-49 to Tyr-60 may comprise the principal site for leukocyte migration activity. A reverse sequence peptide based on Asp-49 to Tyr-60 has no chemotactic action.

The generation of leukocyte chemotactic peptides from HC following limited neutrophil elastase proteolysis suggests a novel function for the amino terminus of this serpin which is not related to serine proteinase inhibition. A schematic diagram illustrating some of the structural features of HC is shown in Fig. 5. The proteinase reactive site region including the reactive site peptide bond of Leu^{444}-Ser^{445} is located near the carboxyl terminus. The putative glycosaminoglycan-binding site is situated in a very basic region of the molecule centered from Lys-173 to Phe-195.

The EuGene PIR protein sequence data banks were searched for human proteins which might contain or partially resemble the chemotactic active site in HC Asp-49 to Tyr-60 (Asp-Trp-Ile-Pro-Glu-Gly-Glu-Glu-Asp-Asp-Asp-Tyr). The

TABLE I

Neutrophil chemotaxis after preincubation with the 19-mer synthetic HC chemotactic peptide

Neutrophils were incubated with or without fMLP or the 19-mer peptide (each at 10^{-8} M for 10 min) prior to testing chemotaxis to fMLP or the HC-neutrophil elastase digest (prepared by reacting the components for 5 min as described in Ref. 13). The concentration of each chemoattractant in the lower wells of the Boyden chambers was 10^{-8} M. Chemotaxis was performed as described under "Experimental Procedures." The data show mean values ± S.E. ($n = 3$).

Pretreatment	% maximal migration to chemotaxin[a]	
	fMLP	HC-neutrophil elastase digest
Medium alone	100	93 ± 22
fMLP	26 ± 7	96 ± 25
19-Mer HC synthetic peptide	87 ± 20	19 ± 7

[a] Results are expressed as the net number of neutrophils migrating as a percentage of the maximal migration to fMLP (47.7 ± 9.0 neutrophils/hpf).

TABLE II

Neutrophil chemotaxis with serpins, prothrombin, proteinases, and their digests

Serpin/prothrombin/proteinase digests were performed as described under "Experimental Procedures" and Ref. 13. The results are reported as the maximum percent chemotaxis (at 10^{-7} to 10^{-8} M chemotactic agent) relative to fMLP and represent at least three separate determinations.

Serpin/protein(ase)/digest	Maximum chemotaxis (as % of fMLP)
Antithrombin (AT)	
AT	0
AT-cathepsin G	7
AT-neutrophil elastase	16
Prothrombin	
Prothrombin	8
Prothrombin-cathepsin G	8
Prothrombin-neutrophil elastase	8
Heparin cofactor II (HC)[a]	
HC	4
HC-cathepsin G	117
HC-neutrophil elastase	48
Proteinase[a]	
Cathepsin G	5
Neutrophil elastase	3

[a] Data for HC and proteinases are taken from Ref. 13.

TABLE III

Migration of mouse neutrophils to HC-neutrophil elastase digest in vivo

The *in vivo* migration activity in mice was performed as described under "Experimental Procedures."

Sample	% neutrophils at 60 min[a]
Medium alone	2.3 ± 2.3
HC control	3.7 ± 3.8
HC-neutrophil elastase digest	8.7 ± 2.1

[a] $n = 3$; paired t test of percent neutrophils in the various samples resulted in a $p < 0.005$ between the HC-neutrophil elastase digest and the medium alone, a $p < 0.025$ between the HC-neutrophil elastase digest and the HC control, and a $p > 0.1$ for the medium alone and the HC control. The total number of peritoneal cells recovered from the medium alone, HC control and HC-neutrophil elastase digest groups was $0.52 \pm 0.20 \times 10^6$, $0.65 \pm 0.35 \times 10^6$, and $0.94 \pm 0.16 \times 10^6$, respectively.

closest sequence matches were high molecular weight kininogen from Asp-586 to Asp-594 (Asp-Trp-Ile-Pro-Asp-Ile-Gln-Thr-Asp) and epidermal growth factor receptor from Trp-586 to Glu-591 (Trp-Ile-Pro-Glu-Gly-Glu). The unique

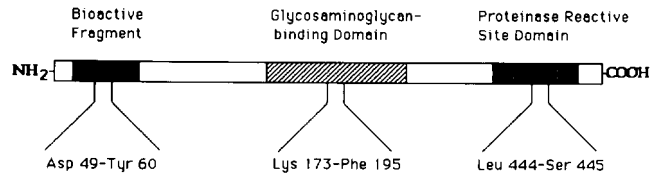

FIG. 5. **Structural features of heparin cofactor II.**

bioactive sequence in HC suggests that generation of this peptide following neutrophil elastase (or cathepsin G) limited proteolysis could be a novel *in vivo* function for this serpin. We have presented preliminary evidence that HC-neutrophil elastase digest products are active *in vivo* in mice. Our data support the hypothesis described by Saroff and Pretorius (30) "that specific biologic functions of some proteins may reside in their unique subsequences with their functions being expressed upon degradation of the parent protein."

Henry (31) demonstrated that leukocytes accumulate in thrombi *in vivo*. Plow (32) showed that active elastase is released from neutrophils during blood coagulation. Kallikrein can also release elastase from neutrophils during thrombus formation (33). Thus, a scenario exists whereby active neutrophil elastase within a thrombus can proteolyze many protein substrates including HC. The physiological concentration of HC is 1.2 μM (34). Maximal activity of the HC peptides *in vitro* is centered around 1 nM, which suggests that only 0.1% of the available HC need be cleaved by neutrophil elastase for generation of physiologically active levels of leukocyte-modulating peptides *in vivo*. Janoff (35) noted that there are several ways for neutrophil elastase to escape its physiological inhibitor, α_1-proteinase inhibitor (α_1-PI): (i) proteinase release in sufficient amounts to overwhelm the inhibitor; (ii) release of proteinase in close proximity to its substrates which then "compete" with the inhibitor; and (iii) proteinase release at sites where inhibitor is inactivated by neutrophil oxidizing systems.

Neutrophil chemotactic activity is generated when α_1-PI, also a serpin, is reacted with neutrophil elastase (36, 37). Neutrophil elastase cleaves α_1-PI at its reactive site and forms a 1:1 complex with the inhibitor which lacks proteolytic activity. By contrast, neutrophil elastase degrades HC to initially yield a molecule which retains thrombin inhibitory activity (16). This HC species is then inactivated by further proteolysis near the reactive site. The chemotactic activity from α_1-PI resides in the reactive site peptide, which remains noncovalently associated with the α_1-PI-neutrophil elastase complex (36, 37). The reactive site peptide derives from the carboxyl terminus of the α_1-PI molecule (36, 37). Synthetic peptides containing portions of the α_1-PI reactive site peptide amino acid sequence also bind to human monocytes and hepatoma cells (38). We have previously shown that the chemotactic activity produced by proteolysis of HC is biologically quite different from that derived from α_1-PI (13–16): (i) α_1-PI-neutrophil elastase complexes are strongly chemotactic only for neutrophils (36, 37), but HC proteolysis products are chemotactic for both neutrophils and monocytes; (ii) chemotactic activity of α_1-PI is localized to the reactive site peptide near the carboxyl terminus (36, 37) while the HC chemotactic activity is juxtaposed to a region near the amino terminus; and (iii) α_1-PI-neutrophil elastase complexes and HC proteolysis products do not bind to the same receptor on neutrophils, based on data from cross-desensitization experiments. These data presented in the current report extend our previous conclusions by demonstrating that the chemoattractant peptides from HC and α_1-PI are not only biologically, but also structurally, distinct.

In summary, our data suggest that evolution has adapted the serpin molecular structure in HC for multiple biologic functions: a region for proteinase active site recognition and inhibition (7, 8, 17)[2]; a region similar to that found in AT for interaction with glycosaminoglycans that greatly enhances the proteinase inhibitory action (7, 8, 40–42); and a region near the amino terminus which releases bioactive peptides, in a manner that is remarkably similar to its serpin homologue angiotensinogen (18, 19). Our results raise the possibility that HC might link coagulation/anticoagulation and inflammation/immune processes. We therefore conclude that HC-derived bioactive peptides may contribute to leukocyte recruitment at sites of thrombosis (31, 32), may modify or influence acute and/or chronic inflammatory responses (11, 12), or may participate in wound healing and tissue repair mechanisms (43).

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[2] Hortin *et al.* (39) recently reported that another synthetic peptide from HC (Gly-54 to Asp-75) is an inhibitor of thrombin fibrinogen clotting activity. The polyanionic composition of this region in HC resembles the carboxyl-terminal region of the antithrombotic compound hirudin, purified from the medicinal leech. In a thrombin-fibrinogen clotting assay, none of the HC synthetic peptides (shown in Fig. 2), at concentrations up to 50 μM, exhibited any ability to inhibit the fibrinogen clotting activity of thrombin (J. L. Woods and F. C. Church, data not included).